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Bcl-2 protein family: Implications in vascular apoptosis and atherosclerosis

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Abstract Apoptosis has been recognized as a central component in the pathogenesis of atherosclerosis, in addition to the other human pathologies such as cancer and diabetes. The pathophysiology of atherosclerosis is complex, involving both apoptosis and proliferation at different phases of its progression. Oxidative modification of lipids and inflammation differentially regulate the apoptotic and proliferative responses of vascular cells during progression of the atherosclerotic lesion. Bcl-2 proteins act as the major regulators of extrinsic and intrinsic apoptosis signalling pathways and more recently it has become evident that they mediate the apoptotic response of vascular cells in response to oxidation and inflammation either in a provocative or an inhibitory mode of action. Here we address Bcl-2 proteins as major therapeutic targets for the treatment of atherosclerosis and underscore the need for the novel preventive and therapeutic interventions against atherosclerosis, which should be designed in the light of molecular mechanisms regulating apoptosis of vascular cells in atherosclerotic lesions.

Keywords Apoptosis · Atherosclerosis · Bcl-2 · Oxidation · Inflammation

Abbreviations

Bcl-2	B cell leukemia/lymphoma-2
Caspases	cysteinyl-directed aspartate-specific proteases
Endo G	endonuclease G
TRADD	TNFR-associated death domain protein
FADD	Fas-associated death domain protein
Daxx	death-associated protein 6
RIP	receptor interacting protein
RAIDD	RIP-associated Protein with a Death Domain
FLIP	FLICE inhibitory protein
cIAP	cellular inhibitor of apoptosis protein-1
SMC	smooth muscle cell

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Introduction

Apoptosis (programmed cell death) is an essential and evolutionary conserved process for normal embryogenesis, organ development, tissue homeostasis and the elimination of deleterious cells from multicellular organisms. Any deregulation or aberrant activation of apoptosis can be involved in the pathogenesis of human diseases such as atherosclerosis, chronic heart failure, cancer, diabetes and neurodegenerative disorders [1–5]. Thus, the cellular and genetic integrity of a cell under stress or oncogenic stimuli have to be strictly controlled to maintain its functionality and viability. The cells are targets of various extrinsic and intrinsic stimuli and they receive and process signals not only from the plasma membrane but also from different compartments within the cytoplasm. This dynamic characteristic of cells enables them to sense signals and response quickly, a fundamental principal of cellular survival. Multiple death and survival signals are integrated to generate molecular apoptotic machinery via protein signaling networks, which are predominantly regulated by the protein-protein interactions, subcellular localization and major protein modifications such as phosphorylation and proteolytic cleavage. An array of protein kinase-mediated pathways targets the major components of apoptotic machinery at the transcriptional and post-translational level and regulates their level and/or function. The balance between pro- and anti-apoptotic signaling

pathways determines the fate of a cell in response to the external or internal stimuli.

The Bcl-2 protein family members have been implicated as the major regulators of the cell death machinery, both positively and negatively [6, 7]. The first member of the family, Bcl-2 was discovered to be overexpressed in low-grade human follicular B cell lymphoma as a result of *t*(14;18) (q32;31) chromosomal translocation [8]. Further genetic studies including overexpression and antisense-mediated suppression of Bcl-2 and molecular characterization approaches including dimerization analysis led to the identification of other members of the family based on conserved BH (Bcl-2 homology) domains and distinguished the Bcl-2 protein family members as the central regulators of apoptotic machinery [9–13]. Despite of intense research on the Bcl-2 protein family, many questions still remain to be answered; when and how are they activated? What are their biochemical activation switches? Do their determined molecular structures help us to characterize their function or are they without information? What may be the clinical implication of these proteins on the treatment strategies targeting apoptosis-related human pathologies?

In this review, we focus on the modulation of human cellular apoptotic pathways by Bcl-2 protein family members and argue on their potential clinical implications within the context of molecular mechanisms of vascular apoptosis in atherosclerosis.

The apoptotic machinery: Guidance to death

Apoptosis is a multi-component programmed cell death process which is characterized by the specific cellular morphological patterns such as chromatin condensation, nuclear fragmentation, cytoplasmic shrinkage, membrane blebbing, the formation of apoptotic vesicles and consequent phagocytosis by immune cells [14]. The molecular changes that occur during apoptosis are the redistribution of phosphotidylserine from the inner to the outer leaflet of the plasma membrane (phosphotidylserine externalization) and internucleosomal DNA cleavage. But how does the apoptotic machinery work? Apoptosis is usually induced by an initiation phase which depends tightly on the cell type and the characteristic of the stimuli (origin, duration, amplitude and the presence of co-stimuli). In a cell under an apoptotic insult either within or outside of the cell, multiple cellular signalling modules are activated synchronously, which can be defined as a battlefield of negative and positive key modulators of apoptosis. The battle is for one decision; should the cell survive or die. During this decisive effector phase, molecular signalling modules serve as the parts of central apoptotic machinery, which should be tightly controlled and finely tuned to maintain the appropriate biochemical functioning of the cell.

Caspases are cysteine-directed, aspartate-specific proteases, which are the main initiators and executioners of the programmed cell death process. Initiator caspases involve caspase-8 and caspase-9, which act upstream of the effector caspases (caspases-3, -6 and -7) [14]. Caspases are normally inactive or minimally active in the unstimulated healthy mammalian cells and they are triggered through a set of signaling events such as activation of a death receptor, a direct DNA damage by chemotherapeutics or cellular stress. Activation of caspase-9 and caspase-3 has been shown to be involved in the ox-LDL-induced apoptosis of vascular endothelial and smooth muscle cells [15, 16, 133]. Furthermore, several reports have demonstrated the involvement of caspase-8 in the apoptosis of vascular SMCs, macrophages and endothelial cells [15, 16, 133]. In macrophages, the activation of caspases-2, -3, -8, and -9 has been shown to be involved in the ox-LDL-induced apoptosis [16]. Besides, the activation of caspases has also been detected in atherosclerotic lesions, which indicates the involvement of caspases in vascular apoptosis [17].

The molecular apoptotic signaling mechanisms in mammalian cells have been a subject of intensive studies for the past few decades and two main pathways with overlapping components have been identified (Fig. 1):

(i) an extrinsic pathway which involves direct initiator cascades triggered by death receptors on cell surface
(ii) an intrinsic pathway which involves mitochondria and intracellular death signals

These two pathways share a couple of adaptor proteins, proteases, protein kinases and protein phosphatases as a part of apoptotic signalling modules, but the potential intersections between these pathways which controls life and death decisions of a cell are not completely identified, yet. A complete up-to-date history of the genomics and proteomics of apoptosis can be found in the excellent reviews published before [18, 19], but a brief outline of the main check points of the extrinsic and intrinsic apoptosis pathways will be described here for a better intervention to describe how the Bcl-2 family proteins function.

The extrinsic or death receptor-mediated pathway is activated in response to the extracellular pro-apoptotic signals and integrated to the apoptotic machinery via specific death receptor adaptors (Fig. 1). The death receptor family members (CD95/Fas/Apo, DR3–6 and TNF-R I-II) are characterized by the presence of cysteine-rich repeats in their extracellular domains and protein-protein interaction modules known as the death domain (DD) in their cytoplasmic portions [20]. Binding of specific ligands induces the receptor multimerization and the formation of a signalling complex known as DISC (death inducing signaling complex), which consists of various adaptor proteins including TRADD, FADD, Daxx, RIP, RAIDD and FLIP [20]. FADD

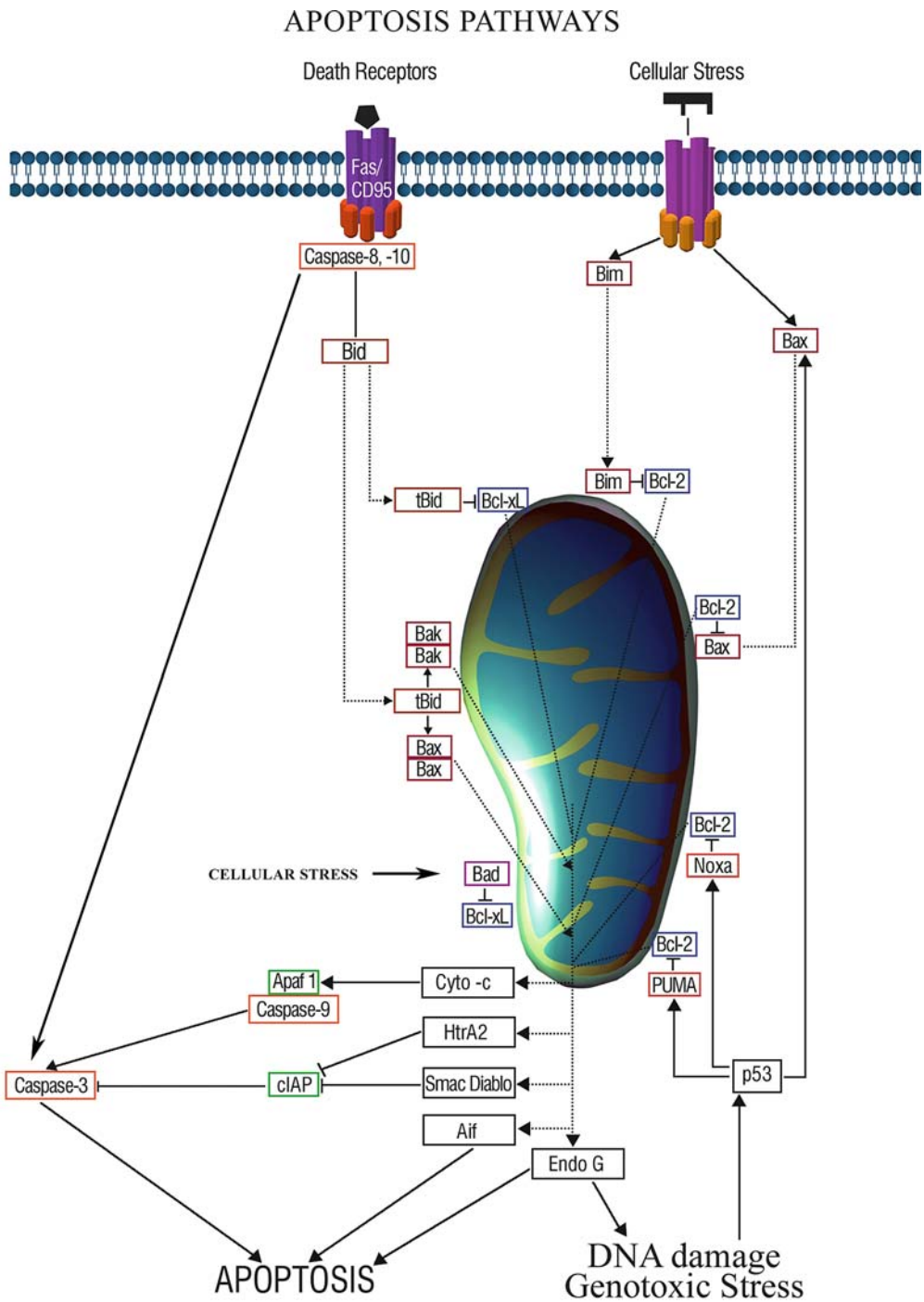

Fig. 1 Two main apoptotic pathways have been described in mammalian cells. The activation of death receptor-mediated (extrinsic) pathway is initiated by binding of a ligand and leads to caspase-8 activation. Once activated, caspase-8 induces either a direct caspase-3-mediated apoptosis (which could be inhibited by cIAP protein) or a mitochondrial amplification loop acting through cleavage and mitochondrial translocation of Bid. Bid (tBid) interacts with pro-apoptotic (Bax, Bak) or anti-apoptotic members (Bcl-2, Bcl-xL) of Bcl-2 family proteins and stimulates the release of cytochrome *c* from mitochondria. Translocation of cytochrome *c* into cytosol leads to the constitution of a protein complex (apoptosome) which facilitates caspase-9 activation and apoptosis. The intrinsic (mitochondrial) apoptosis pathway is activated upon cellular and genotoxic stress such as lipid peroxidation and oxidative stress, growth factor withdrawal or UV radiation. Direct involvement of Bcl-2 proteins enables integration and interpretation of apoptotic or survival signals originating either from extracellular or intracellular stimuli. Multidomain pro-apoptotic Bcl-2 proteins Bak and Bax form channels on mitochondria and facilitate the release of apoptosis regulator proteins (cytochrome *c*, HtrA2, Smac/DIABLO, AIF) while multidomain anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL) inhibit the release of these apoptosis regulator proteins. BH3-only pro-apoptotic Bcl-2 proteins (Bad, Bim, Bid, Noxa, and Puma) selectively interact either with multidomain pro-apoptotic (direct activators) or anti-apoptotic (sensitizers) Bcl-2 proteins and promote apoptosis. Smac/DIABLO and HtrA2 prevents cIAP-1-mediated inhibition of caspase-3 and facilitate the progression of apoptosis. (See text for further details)

acts as a bridge between DISC and caspase-8, which is critical for the recruitment and oligomerization of caspase-8 in the DISC, as well as the autocatalytic activation of caspase-8 and the activation of death receptor-mediated programmed cell death [21–23].

The direct activation of the effector caspases-3 and -7 by caspase-8 may not necessarily involve the mitochondrial events; however in many cell types death receptor-mediated apoptotic signalling utilizes a mitochondrial death amplification loop [21, 24, 25]. The reason for this preference has not been identified clearly, but insufficient amount of active caspases or the abundance of downstream inhibitors of the apoptotic machinery was suggested to be involved in this paradigm. The mitochondrial amplification loop involves the caspase-8-mediated cleavage of the cytosolic BH3-only pro-apoptotic Bcl-2 family member, Bid, which represents an integration of two apoptotic pathways on mitochondria (Fig. 1). Upon processing by caspase-8, Bid translocates from cytosol to mitochondria where it oligomerizes with pro-apoptotic Bcl-2 family members Bax and Bak and mediates cytochrome *c* release [14, 18, 25]. The cytosolic cytochrome *c* induces the formation of the apoptosome complex, which is composed of seven Apaf-1 (Apoptotic protease activating factor-1) molecules, each bound to one molecule of cytochrome *c* and a dimer of caspase-9. Formation of the apoptosome results in the activation of caspase-9, which thereby activates the effector caspases-3 and -7 to initiate the execution of apoptosis [18, 25]. The intracellular components which convey the apoptotic stimuli to the central apoptotic machinery are not identified completely, but there is one reality that has been shown clearly; mitochondria lies in the center of apoptotic machinery in most cases.

The intrinsic apoptosis pathway involves direct and active contribution of mitochondria. This pathway is initiated by receptor-independent apoptotic stimuli such as DNA-damaging agents, UV and γ-radiation, hypoxia and growth factor withdrawal [26–29]. These stimuli target the intracellular signalling components which transmit the apoptotic signal to the main apoptotic machinery.

In mammalian cells Bcl-2 family proteins are one of the main "apoptotic sensors" mentioned above and they act primarily on the mitochondria, where they regulate the survival or death signals in a preventive or provocative fashion. Upon exposure to apoptotic insults many apoptosis regulator proteins such as cytochrome *c*, SMAC (second mitochondria-derived activator of caspases)/DIABLO (direct inhibitor of apoptosis-binding protein with low pI) and Omi/HtrA2 (high-temperature-requirement protein) are released from the mitochondria (Fig. 1) [18, 30–32, 133]. Additionally, proteins responsible for the caspase-independent DNA fragmentation and apoptosis-like nuclear morphology (apoptosis inducing factor (AIF) and endonuclease G) are also released from the mitochondria following apoptotic stimuli [33]. Increased AIF expression has been shown to regulate the ox-LDL-induced apoptosis in human coronary artery endothelial cells [34]. Thus, mitochondrial integrity is critical for maintaining the cellular homeostasis and proper compartmentalization of the apoptotic mediators. The mechanisms for the intrinsic apoptosis pathway and induction of mitochondrial permeabilization are not completely understood, but recent studies provide some clues about how apoptotic stimuli induce the permeabilization of mitochondrial membranes.

There is more than one model for the cytosolic escape of mitochondrial proteins in response to apoptotic stimuli (Fig. 2):

Permeability transition pore opening

The permeability transition pore (PTP) complex is a large polyprotein channel mainly formed by the mitochondrial outer membrane voltage-dependent anion channel (VDAC) and the mitochondrial inner membrane protein adenine nucleotide translocase (ANT). The PTP structure also includes regulatory components such as mitochondrial matrix protein cyclophilin D, peripheral benzodiazepine receptor, hexokinase II and creatine kinase. PTP is suggested to span both outer and inner mitochondrial membranes and its opening is characterized by mitochondrial depolarization, depletion of ATP and the release of Ca^{2+} from the mitochondrial matrix followed by the swelling of the mitochondrial matrix [35, 36]. The total surface area of inner mitochondrial membrane is greater than the outer membrane; hence an uncontrolled expansion of the inner mitochondrial membrane may lead to the rupture of the outer mitochondrial membrane and the release of intermembrane proteins to cytosol. The role of the PTP model in apoptosis has not been confirmed; a transient opening of the PTP and flicking between open and closed states have been proposed to allow cytochrome *c* to be released although ATP production and mitochondrial integrity are preserved [37, 38]. Even if this transient opening may lead to the release of some components out of mitochondria, the PTP model does not seem to completely explain the structural perturbations of mitochondria during the apoptotic process.

Bcl-2 family members interact with outer mitochondrial membrane proteins

The pore size of PTP itself is only sufficient to allow the passage of molecules as large as 1.5 kDa, which is not sufficient enough to allow the passage of cytochrome *c*. Therefore, it has been suggested that the interaction of Bcl-2 family proteins with components of PTP can either increase or decrease pore size and regulate the mitochondrial permeability

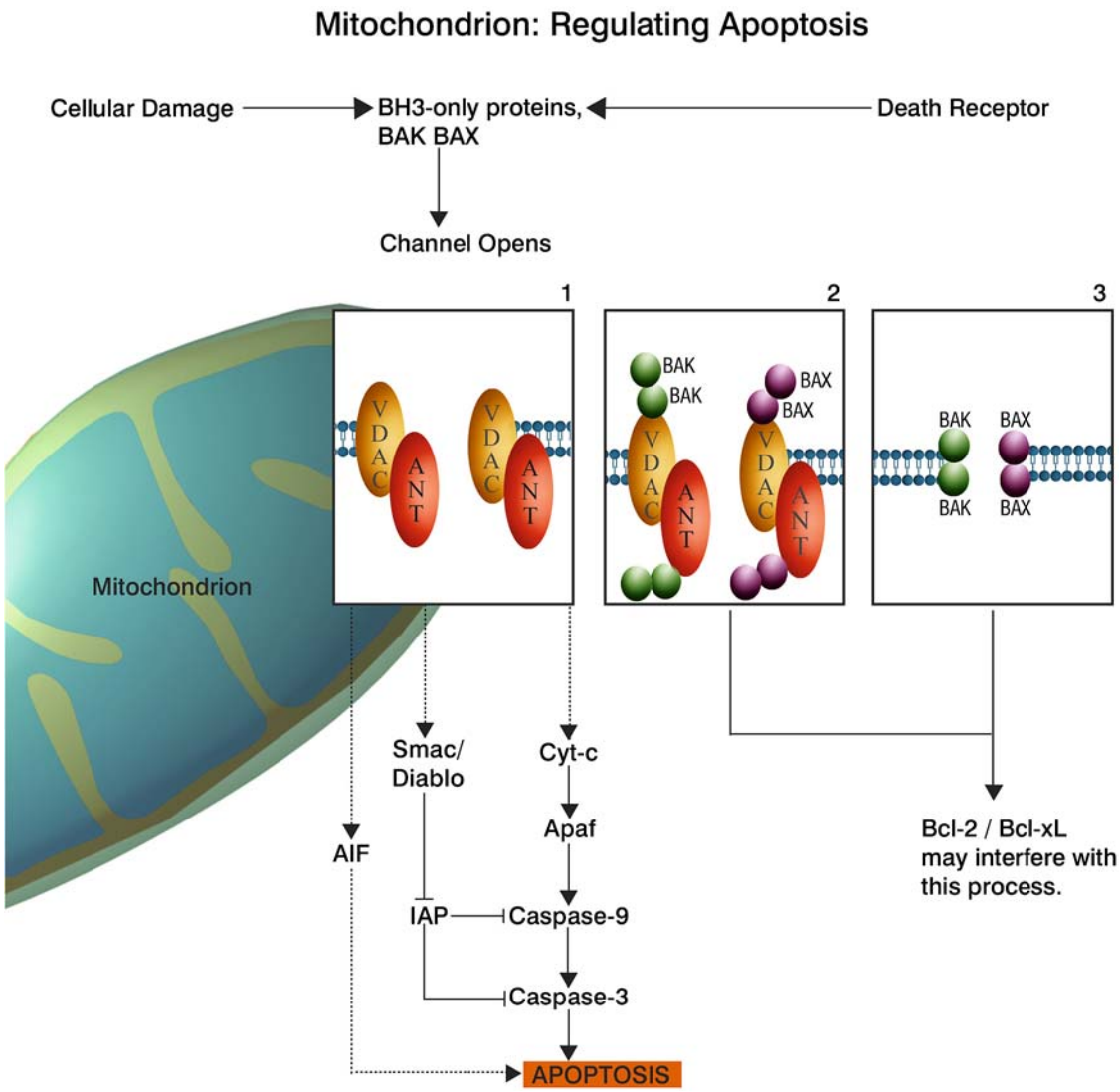

Fig. 2 Three models for the cytosolic escape of mitochondrial proteins in response to apoptotic stimuli. Upon an apoptotic stimulus such as cellular stress (oxidative stress, UV irradiation, growth factor withdrawal) or activation of death receptors, a mitochondrial channel could be formed by: 1. Voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT), 2. VDAC-ANT-Bcl-2 proteins (Bax, Bak), 3. only Bcl-2 proteins (Bax, Bak). Anti-apoptotic members of Bcl-2 protein family (Bcl-2, Bcl-xL) may interfere with the apoptotic process through the inhibition of cytosolic escape of mitochondrial proteins

[39, 40]. The pro-apoptotic Bax protein has been shown to interact with ANT and promote pore opening [41]. In contrast, anti-apoptotic Bcl-2 and Bcl-xL proteins counteract pore opening and thereby prevent cytochrome *c* release through protein-protein interactions with PTP components [42, 43]. Additionally, it has been shown that Bax and Bak may bind directly to VDAC and keep the pore in a sustained open state that allows cytochrome *c* leakage, while Bcl-xL may lock the VDAC in a cytochrome *c* impermeant structure to prevent the cytochrome *c* leakage [40, 44]. Although many Bcl-2 proteins are co-immunopurified with some components of PTP, the exact mechanisms of regulation and the role of structural constraints on these mechanisms still remains to be elucidated.

Bcl-2 family members as channel forming proteins

The release of proteins from mitochondrial intermembrane space has been suggested to be mediated by pores large enough to allow the passage of intermembrane proteins into the cytosol. These pores have been reported to be formed by Bcl-2 family members through their transmembrane domains [45–48]. Bcl-2 family proteins were shown to insert themselves into synthetic lipid bilayers, which is followed by oligomerization and channel formation [46, 48]. This crucial step is regulated either in a provocative or inhibitory manner, which has been proposed to depend on the structural differences of transmembrane domains that form the channel [49–51]. This fact determines the molecular behavior

of protein-transport pores in the outer mitochondrial membrane.

Bcl-2 proteins: regulating apoptosis

The multidomain anti-apoptotic Bcl-2 proteins

The Bcl-2 family proteins can be classified into three groups based on their structural and functional properties (Fig. 3). The first group involves the multidomain anti-apoptotic members Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl-1, Boo/Diva and NR-13. They exhibit all four Bcl-2 homology domains (BH1-4) which are essential for their survival function through mediating the protein-protein interactions and a transmembrane domain which is formed by a stretch of hydrophobic amino acids near to their C-terminal. The C-terminal domain is required for anchoring or insertion into the cellular membranes of not only mitochondria but also nucleus and endoplasmic reticulum [52, 53]. The α-helices of BH1, BH2 and BH3 domains form a hydrophobic pocket and the N-terminal BH4 domain further stabilizes this structure [54–56]. The protein structure of Bcl-xL complexed with the BH3 domain of Bak suggested a functional interaction of the amphiphathic α-helix of Bak BH3 with the hydrophobic groove formed by BH1-3 domains of Bcl-xL [57]. Both BH3-only and multidomain pro-apoptotic Bcl-2 proteins appear to act through the exposure of their BH3 domains following an apoptotic insult [58–60]. This protein-protein interaction model proposed that the anti-apoptotic Bcl-2 members act as the functional traps of pro-apoptotic members, but is the cellular machinery always in a pro-apoptotic conditioning, which should be continuously blocked by anti-apoptotic Bcl-2 members? Do the pro-apoptotic members, which are present ubiquitously in the local cellular compartments, pro-actively sequester the

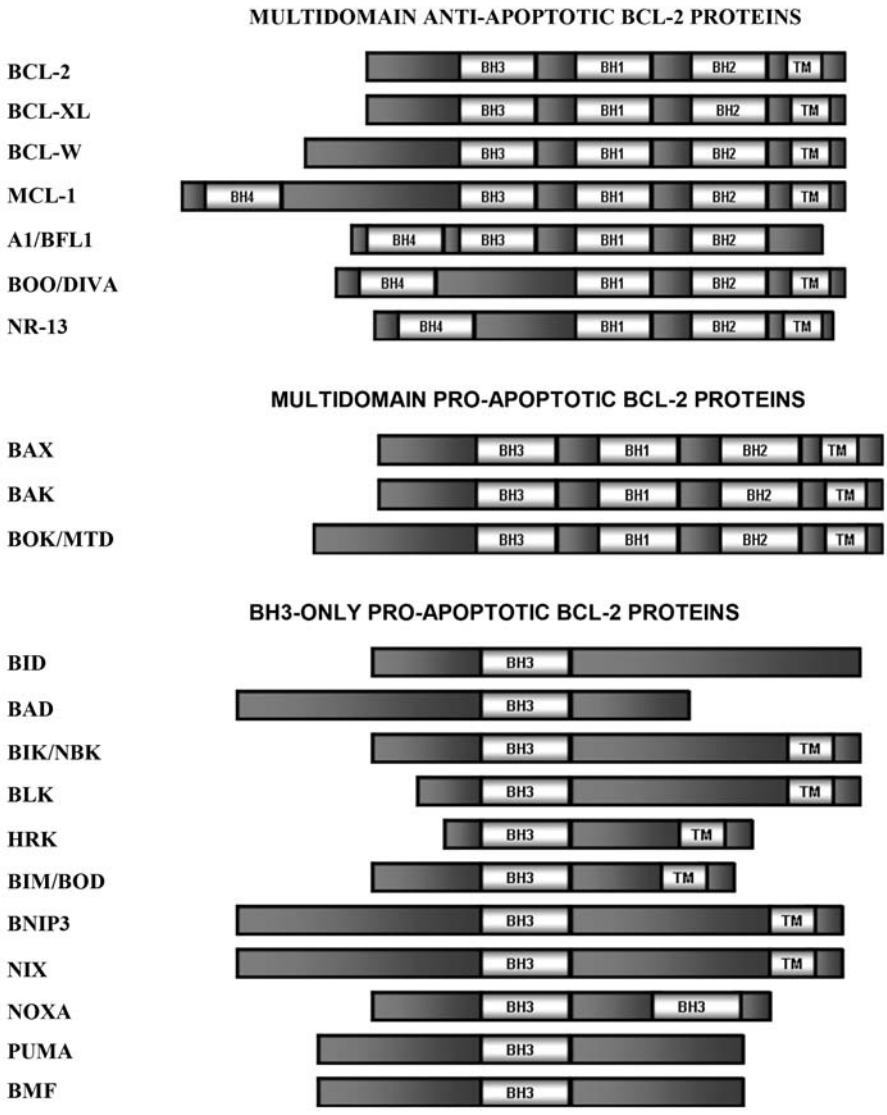

Fig. 3 Classification of Bcl-2 protein family members

"silencers" of apoptotic machinery? Even though the exact biochemical mechanisms for the actions of anti-apoptotic Bcl-2 members remain to be elucidated, the efforts to clarify these mechanisms provided us many clues. Bcl-2 has been shown to modulate cellular viability through regulating intracellular calcium homeostasis, cellular redox state, lipid peroxidation, as well as cytochrome *c* release from mitochondria [61–64]. Bcl-2 has been shown to attenuate apoptosis induced by ionizing radiation, chemotherapy, UV radiation and death receptors [27, 65–67]. Overexpression of Bcl-2 protects SW480 cells from TRAIL-induced apoptosis via the attenuation of caspase-8 activation and the cleavage of Bid and caspase-3 [68]. In contrast, Fas/FasL-, TRAIL- and TNFα-mediated apoptosis pathways have been proposed to be insensitive to the blockage by Bcl-2/Bcl-xL [69–71]; thereby the exact contribution of Bcl-2/Bcl-xL in the receptor-mediated extrinsic apoptotic pathway remains undetermined. Mice deficient in Bcl-2 have been demonstrated to have gray hair, polycystic kidneys and decreased number of lymphocytes [72]. Mice deficient in Bcl-xL die at around day 13 of the gestation due to massive neuronal and hematopoetic apoptosis [73]. Bcl-xL and Bcl-2 were shown to inhibit the apoptosome formation by preferentially sequestering Apaf-1, but these proposed interactions have yet to be confirmed [74]. Bcl-2 and Bcl-xL were also reported to abrogate the mitochondrial translocation and oligomerization of Bax in the outer mitochondrial membrane [75, 76]. Moreover, they have been shown to interfere with the oligomerization and activation of Bak [77, 78].

The mechanistic insight, which is derived from the cases characterized with Bcl-2 overexpression, remains insufficient to explain the role of Bcl-2 on resistance to apoptosis. In addition to the level of expression, post-translational modifications such as phosphorylation and proteolytic cleavage may regulate the activity of Bcl-2 and Bcl-xL. Phosphorylation of Bcl-2 at Ser-70 by PKC has been reported to be required for its anti-apoptotic function [79]. In contrast, microtubule-targeting agents such as paclitaxel have been shown to induce hyperphosphorylation of Bcl-2 (Ser-70, Ser-87 and Thr-69) and abrogate its anti-apoptotic effect [80]. Phosphorylation of Bcl-xL at Ser-62 by JNK (c-Jun N-terminal kinase) in response to taxol or 2-methoxyestradiol treatment has been reported to oppose the anti-apoptotic function of Bcl-xL and sensitizes prostate cancer cells to apoptosis [81]. The caspase-dependent N-terminal cleavage of Bcl-2/Bcl-xL and the resulting exposure of their BH3 domains converts these anti-apoptotic proteins into pro-apoptotic ones. Chemotherapy-induced cleavage of Bcl-2 is mediated by caspases and results in the formation of a 23 kDa fragment of Bcl-2 [82]. In PC12 pheochromocytoma cells cleavage of Bcl-2 was detected in neocarzinostatin-induced but not in cisplatin-induced apoptosis, which indicates a stimuli-dependent modification of Bcl-2 [83]. The cleavage site of Bcl-2 by caspase-3 has been mapped at the loop domain of Bcl-2 at Asp-34 and the C-terminal of the cleavage product was shown to mediate apoptosis through its BH3 homology and transmembrane domain [84]. Caspase-3-dependent cleavage of Bcl-2 appears to further amplify the caspase activation pathway and act as a positive feedback mechanism in the apoptotic signaling [85]. Bcl-xL has been shown to be cleaved by caspase-3 after Asp-61/Asp-76 and by calpain after Ala-60 and these cleaved products have been shown to form the cytochrome *c*-releasing channels in lipid membranes [86].

The multidomain pro-apoptotic members of Bcl-2 protein family

This second group of Bcl-2 protein family mainly involves Bax, Bak and Bok/Mtd (Fig. 3). Bax is mainly localized in the cytosol or loosely attached to the outer membrane of mitochondria or ER as a monomer. Following an apoptotic stimuli, Bax undergoes a unique conformational change exposing its C-terminal hydrophobic domain, which is involved in its anchorage to the mitochondrial membrane [87]. In the mitochondrial membranes, Bax forms dimers, oligomers or high-order multimers [45]. Another important multidomain proapoptotic Bcl-2 protein family member is Bak, which is an integral protein of the outer mitochondrial membrane and ER. Similar to Bax, Bak also undergoes a conformational change -the open conformer- in response to apoptotic stimuli such as etoposide and cisplatin [88, 89]. The inhibitory effect of Bcl-2 on Bak acts through selective interaction of Bcl-2 with the open conformer (N-terminal exposed conformation) of Bak [77]. The principles of these conformational changes and oligomerization of Bak and Bax proteins remain to be explained at structural level. The involvement of Bak and Bax in apoptosis regulation is demonstrated by the insensitivity of Bak $-/-$ Bax $-/-$ MEFs to multiple apoptotic stimuli including chemotherapeutics and UV radiation [90]. Afterwards, the requirement of Bak and Bax in the apoptotic machinery has been confirmed by many studies. Bax null cells have been shown to be resistant against TRAIL-induced apoptosis [91]. Bax deficiency did not effect the processing of caspase-8 or Bid cleavage by TRAIL, but the release of Smac/DIABLO which is required for the inhibition of IAP proteins and caspase-3 activation was abrogated [91]. In TRAIL-resistant leukemic cells that are deficient in Bax and Bak, the release of mitochondrial proteins appear to be abrogated and the adenoviral transduction of the Bax gene, but not the Bak gene, to the Bax/Bak-deficient leukemic cells rendered them TRAIL-sensitive as assessed by the enhanced apoptotic death and caspase-3 processing [92]. Recently, the activation of multiple caspases by DNA damage and ER stress has been shown to be directly regulated by Bax and Bak in double knock-out MEFs [93]. Post-translational

modifications of Bax or Bak such as proteolytic cleavage have been shown to regulate the functional impact of these proteins on apoptosis. Calpain-mediated conversion of Bax into a truncated form (arises from cleavage of N-terminal 33 amino acids, p18 Bax) enhances its pro-apoptotic properties upon stimulation with chemotherapeutics [94]. After truncation into its p18 form, Bax behaves like a BH3-only protein and the potentiation of apoptosis by p18 Bax has been proposed to be related to its increased affinity for Bcl-xL. Furthermore, a cathepsin-like cysteine protease is involved in the degradation of p18 Bax and the stabilization of p18 Bax by cathepsin inhibitors enhances the drug-induced apoptosis [95].

BH3-only members of Bcl-2 protein family

The third group of the family involves BH3-only proteins such as Bid, Bad, Bim, Bik, Blk, Hrk, BNIP3, Nix, BMF, Noxa and Puma (Fig. 3). These proteins share only the amphipathic α-helical BH2 homology domain and mainly act through inhibition of Bcl-2/Bcl-xL and activation of Bak and Bax. They act as the sentinels of cell death sensing machinery and they coordinate the fine-tuning of apoptotic response through their interactions with pro- and anti-apoptotic Bcl-2 members. This fine-tuning phenomenon has been attributed to the selective interaction of certain BH3-only proteins with either anti-apoptotic or pro-apoptotic Bcl-2 proteins, but the definitive mechanisms that lie behind this phenomenon remain to be clarified [96]. There are two main pathways which characterize the function of BH3-only proteins on mitochondria (Fig. 1);

(i) Direct activators: Some BH3-only members (Bid and Bim) interact with the pro-apoptotic Bcl-2 proteins such as Bak and Bax and thereby induce their activation/oligomerization. This type of activity of BH3-only proteins can be attenuated by Bcl-2 through selective sequestration and functional silencing
(ii) Sensitizers: Other BH3-only members (Bad) interact with the anti-apoptotic Bcl-2 proteins and prevent them binding and sequestering BH3-only members such as Bid and Bim, which can activate Bak and Bax.

The functional regulation of BH3-only proteins at the cellular level could be regulated by;

(a) Phosphorylation

Selective phosphorylation of proteins at the different residues may modulate different molecular and cellular responses. For example, survival signals have been shown to induce the phosphorylation of Bad on the Ser-112, Ser-136, and Ser-155, which leads to the sequestration and inactivation of Bad by 14-3-3 proteins [97, 98]. Recently, a novel Cdc2- or JNK-mediated phosphorylation site of Bad has been mapped at the Ser-128 and this modification has been demonstrated to inhibit the sequestration of Bad by members of 14-3-3 family and enhance its pro-apoptotic effect [99, 100]. Cytokine-dependent phosphorylation of Ser-170 has been demonstrated to negatively regulate the pro-apoptotic activity of Bad [101]. Furthermore, phosphorylation of Bim at Ser-65 by JNK has been shown to mediate the trophic factor withdrawal-induced Bax-dependent apoptosis [102]. Phosphorylation of Bad has also been shown to be mediated by protein phosphatases. Free fatty acids (oleic acid and linoleic acid as major components) released from lipoproteins in response to the lipoprotein lipase treatment have been shown to induce apoptosis through the activation of protein phosphatase type 2Cbeta (PP2Cβ) and the dephosphorylation of Bad on Ser-112 [141].

(b) Transcriptional control

Puma (p53 up-regulated modulator of apoptosis) and Noxa are the transcriptional targets for p53 [103, 104]. PUMA is transcriptionally induced by the chemotherapeutics 5-FU (5-Fluorouracil) and Adriamycin in a p53-dependent fashion and it is localized to mitochondria where it interacts with Bcl-2 and Bcl-XL through its BH3 domain [105]. In contrast to Noxa, the pro-apoptotic effect of Puma has been shown to depend on the conformational change and the multimerization of Bax [106]. Induction of Noxa did not show any relevance to the subcellular localization of Bax, but it selectively interacts with Bcl-2, Bcl-xL and Mcl-1 via its BH3-only domain [104].

(c) Cleavage

Following death receptor signalling, the full length 22 kDa Bid is cleaved within its unstructured loop and a 15 kDa truncated form of Bid is created, tBid [107, 108]. Cleavage of Bid results in exposure of a new terminal glycine residue which is N-myristoylated [109]. Upon N-myristoylation, tBid is selectively routed to mitochondria and induces oligomerization of Bax and Bak.

Role of apoptosis in atherosclerosis

Atherosclerosis and its complications such as acute myocardial infarction and stroke are the leading cause of mortality and morbidity in the industrialized and developing countries [110]. The pathophysiology of atherosclerosis is characterized by an initial fatty streak formation, which progresses by alteration of endothelial function, expression of adhesion molecules, inflammatory response as well as lipid retention/oxidation and engulfment by macrophages which forms

Table 1 A summary of the implications of the Bcl-2 protein family in vascular apoptosis and atherosclerosis

Cell type	Pathological implication	Involvement of Bcl-2 protein family
Endothelial cells	Apoptosis of endothelial cells leads to endothelial dysfunction, increased risk of thrombosis and atherosclerotic lesion progression	↑ Bax[121,122], Bak[122], Bad[139] expression, Bax/Bcl-2 ratio[125,135] ↓ Bcl-2[121,125,127,133,139], Bcl-xl[122,127,139] expression
Smooth muscle cells	Apoptosis of smooth muscle cells leads to the destabilization and rupture of the fibrous plaque and increased risk for thrombosis.	↑ Bax[121,122], Bak[122] expression ↓ Bcl-2[122] and Bcl-xl[122] expression
	The proliferation and intimal migration of vascular smooth muscle cells result in increased synthesis of collagen and formation of fibrous plaques	↑ Bcl-xl[123] expression
Macrophages	Apoptosis of macrophages leads to formation of highly instable cellular structures of the plaque as a result of the necrosis and apoptosis.	↑ Bax[121,122,136], Bak[122] expression, activation of Bad and Bim[138] ↓ Bcl-2[121] and Bcl-xl[138] expression,

one of the most characteristic hallmarks of atherosclerosis: foam cells [111]. The cell proliferation, apoptosis and migration contribute to the progression of atherosclerotic lesion (Table 1). The proliferation and intimal migration of vascular smooth muscle cells results in the increased synthesis of collagen and the formation of fibrous plaques around lipid cores which are mainly formed by lipid-laden macrophages. In advanced lesions, the central parts of these lipid cores become highly instable as a result of the necrosis and apoptosis of cellular structures of the lipid core. The rupture of the fibrous plaque occurs usually at the shoulder region of the lesion, which is followed mostly by the formation of a thrombus and the increased risk of acute coronary syndromes and stroke (Table 1).

So what is the role of apoptosis in the mechanistic development of atherosclerosis? The progression of an atherosclerotic lesion is evolutionary and chaotic, but we can describe the pattern of lesion development into two main phases with overlapping characteristics. The first phase is defined by the endothelial dysfunction and inflammation with prominent lipid retention but minimal lipid peroxidation [110, 112]. This pro-inflammatory microenvironment predominantly provokes a proliferative response for vascular smooth muscle cells, which is followed by intimal migration and neointima formation [113, 114]. It has been also speculated that endothelial cell apoptosis has a role in the development of endothelial cell dysfunction and atherosclerotic lesion areas show an extensive endothelial cell turn-over with dysfunctional endothelial cells [115]. Thereby, functional involvement of apoptosis in the first phase of lesion development targets two cell types: a preferential pro-apoptotic stimulation for endothelial cells and an anti-apoptotic and proliferative stimulation for smooth muscle cells. The second phase of lesion progression involves an increased inflammatory response and lipid retention/peroxidation, which trigger the formation of oxidized low density lipoprotein (ox-LDL) particles in the vascular wall, an alteration of redox balance and the modification of cellular proteins, DNA and lipids [111]. This leads to the plaque development and stabilization of the plaque by the extracellular matrix and the cellular support formed by vascular smooth muscle cells. In more advanced lesions, extensive apoptosis of cells which form the plaque (lipid-laden macrophages and SMCs) may lead to the thinning of fibrous support, plaque destabilization, rupture and thrombosis, which may result in the clinical presentation of the lesion such as acute myocardial infarction [116, 117]. In the second phase of lesion development, there are mainly two cell types as the targets for pro-apoptotic insults: macrophages and SMCs. Therefore, therapeutic strategies aiming the prevention and the treatment of atherosclerosis should be designed in the light of apoptotic mechanisms unique for the evolutionary phase of vascular lesion. Induction of apoptosis of SMCs to prevent the progressive thickening of vascular wall and the protection of endothelial cells against apoptosis at the same time may be beneficial in the initial phase of atherosclerotic lesion development. Moreover, selective induction of apoptosis in inflammatory cells residing in the vascular wall could prevent the lesion progression or interventions to prevent plaque rupture by abrogating SMCs or macrophage apoptosis may be possible for the prevention of clinical presentation. The complexity of these interventions could be overcome

if the molecular and cellular mechanisms that regulate apoptosis in different vascular cells in the particular phases of atherosclerotic lesion development are identified. Bcl-2 family protein members potentially lie in the heart of these mechanisms.

Bcl-2 family proteins: role in vascular apoptosis?

As mentioned previously, the signal transduction pathways which are involved in vascular apoptosis dictate the regulatory interactions between anti- and pro-apoptotic members of Bcl-2 family as well as the mitochondrial leakage and activation of caspases.

Both extrinsic and intrinsic apoptosis pathways have been assumed to play role in vascular apoptosis involved in atherosclerosis progression. Although apoptosis of vascular cells in response to the activation of extrinsic death signaling modules such as Fas (CD95), and tumor necrosis factor family receptors have been demonstrated [118–120], the involvement of Bcl-2 protein family members in this process has not been characterized. The apoptotic process in different stages of atherosclerotic lesions has been studied in human atherosclerotic plaques from whole-mount carotid endarterectomy specimens and advanced atherosclerotic plaques were characterized by an extensive loss of SMCs and the presence of lipid-laden macrophages [121]. These lipid-laden macrophages show an increase in the expression of the pro-apoptotic protein Bax, but not the anti-apoptotic Bcl-2. In these lesions, fairly low numbers of SMCs were detected around the necrotic core and these lipid-laden SMCs were also demonstrated to express a strong cytoplasmic Bax expression [121]. The cellular structures in fatty streaks were also characterized by the expression of Bax in contrast to the cells in adaptive thickening with no Bax expression. In another study, expression of Bcl-2 family proteins has been investigated in endarterectomy and atherectomy specimens from renal, coronary and carotid arteries [122]. SMCs were found to be the primary cell type undergoing apoptosis and in all apoptotic cells Bax and Bak expression was present, while Bcl-xL and Bcl-2 was missing in the majority of apoptotic cells. In advanced lesions, non-apoptotic cells were shown to express higher Bcl-xL levels than control specimens, which may be an induced protective response against apoptotic insults. There was no difference in Bcl-2 expression between non-apoptotic cells in healthy and advanced atherosclerotic plaques, which points out Bcl-xL as the primary "inducible" protective factor in vascular apoptosis *in vivo* and the loss of basal Bcl-2 expression in vascular cells as an initiative factor for the susceptibility of vascular cells to apoptosis [122]. Increased levels of Bcl-xL have been observed in the intima of early proliferative lesions in a rabbit atherosclerotic model and the downregulation of Bcl-xL levels in neointima by antisense oligonucleotides resulted in apoptosis of vascular cells and the regression of lesions, which indicates the requirement to develop specific treatment strategies for specific stages of atherosclerosis [123].

The mechanistic role of the expression pattern of Bax in arterial apoptosis has been partially characterized. Bax has been shown to be present in normal intimal and medial SMCs in some studies [123, 124]. Up-regulation or post-translational modification of Bax, which may create different kinetics of interactions between Bcl-2 family members on mitochondria may regulate the apoptotic response, but this phenomena still remains to be identified. The selective expression of Bax and Bak without expresion of Bcl-xL and Bcl-2 in apoptotic cells, but not in non-apoptotic cells of advanced plaques underlines the importance of these proteins in vascular apoptosis and these findings provide valuable data on the possible modulatory effect of Bcl-2 proteins on the atherosclerotic lesion progression.

The crosstalk between the inflammatory reactions and lipid peroxidation may promote the initial atherosclerotic lesion progression [111], as well as an apoptotic re-shaping of cellular structures in advanced lesions. Bcl-2 expression was decreased in primary endothelial cells after treatment with IFN-γ and to a lesser degree when treated with TNF-α. Moreover, treatment with combination of IFN-γ and TNF-α resulted in a more pronounced downregulation of Bcl-2 [125]. An upregulation of Bax was reported in response to both cytokines and the Bcl-2/Bax ratio was proposed to determine the apoptotic effect of cytokines on primary endothelial cells (HUVECs). TNF-α-induced apoptotic response in glomerular endothelial cells has been demonstrated to be mediated by the upregulation of Bak and downregulation of Bcl-xL [126]. Furthermore, overexpression of Bcl-2 and Bcl-xL was demonstrated to protect endothelial cells from TNF-α-induced apoptosis in another study [127]. Interestingly, inhibition of Bcl-xL expression by anti-sense interventions sensitized HUVEC cells to either ceramide or staurosporin and Bcl-xL has been proposed to protect endothelial cells against the caspase-dependent and caspase-independent mechanisms of mitochondrial membrane disruption [128]. Another Bcl-2 family member protein, A1 was shown to be increased upon TNF-α- treatment but downregulation of A1 by anti-sense intervention did not have any effect on apoptotic response [128]. In a recent study, p38 MAP kinase pathway was shown to regulate TNF-α-induced apoptosis in endothelial cells via phosphorylation and down regulation of Bcl-xL, which presented Bcl-xL as a major anti-apoptotic factor against the cytokine-induced receptor mediated apoptosis in endothelial cells [129]. Additionally, Kim et al. have shown that proteasomal inhibition enhances TNF-α-induced cell death in SMCs, but this effect is independent of cytochrome *c* release and Bcl-2 proteins [130]. Ox-LDL

may induce apoptosis in vascular cells through the activation of Fas-FasL pathway and FasL is expressed on the vascular endothelium and it may induce apoptosis in Fas-expressing cells in the vascular wall [131, 132]. Although vascular endothelial cells are resistant to the Fas-mediated apoptosis under physiologic conditions, ox-LDL load may sensitize them to apoptosis since ox-LDL-induced apoptosis of aortic endothelial cells cultured from Fas −/− & FasL −/− mice has been shown to decrease when compared to the wild-type mice [131]. In a recent study, ox-LDL has been shown to directly activate the intrinsic apoptotic pathway in human coronary endothelial cells without the activation of caspase-8 and the truncation of Bid leading to a decrease in the expression of Bcl-2 and c-IAP antiapoptotic proteins [133]. Overexpression of Bcl-2 was also shown to inhibit the ox-LDL-induced apoptosis in U937 macrophages [134]. Ox-LDL was shown to increase Bax/Bcl-2 ratio and thereby promoting the susceptibility of vascular cells to apoptosis [135]. Furthermore, loading of macrophages with free cholesterol was proven to induce both Fas- and mitochondria-mediated apoptosis and increased the cytoplasmic and mitochondrial Bax levels through a post-translational modification [136]. Again in a novel study, it has been clearly demonstrated that the reduction of macrophage apoptosis stimulated atherosclerosis in LDL-R −/− mice. The aortic lesion area has been found to be more prominent in LDL-R −/− mice reconstituted with Bax −/− bone marrow after irradiation [137]. Oxysterols have been reported to induce apoptosis in murine macrophages through degradation of protein kinase B/Akt, activation of BH3-only proapoptotic proteins Bad and Bim, and downregulation of Bcl-xL [138]. Furthermore, siRNA knockdown of Bax led to a complete blockage of 25-hydroxycholesterol-mediated apoptosis in these cells.

The apoptotic pathways induced by mildly oxidized LDL in primary cultures of human coronary endothelial and SMCs were evaluated [139]. It has been demonstrated that apoptotic signals were mediated by the extrinsic pathway mainly through Fas and TNF-R I-II receptors. In endothelial cells a prominent decrease in Bcl-2 and an increase in proapoptotic Bad protein but no changes in Bax protein levels were detected in response to mildly oxidized LDL [139]. Additionally, no noticeable changes have been observed in the Bcl-2 protein family members in SMCs treated with mildly oxidized LDL. Bcl-2 expression has also been proposed to modulate the cellular balance between apoptosis and necrosis in response to ox-LDL treatment instead of prevention. Interestingly Bcl-2 expression seems to potentiate the toxic effects of ox-LDL and thereby may lead to extensive necrosis and plaque destabilization [140]. All these contradictory results underline the need for further research efforts aiming to identify the major components in vascular apoptosis.

Conclusions

Atherosclerosis is still the leading cause of mortality and morbidity in industrialized societies despite of the preventive and curative measures. There should be one important notification to be emphasized; a higher apoptotic index is observed in advanced atherosclerotic lesions compared to early lesions, and the role of apoptosis in atherosclerosis pathogenesis should be defined clearly for each specific phase of the vascular lesion development. The lack of available data in literature on the role of Bcl-2 family members in vascular apoptosis in atherosclerosis models is one of the major limitations for development of novel therapeutic strategies against treatment and prevention of atherosclerosis. Identification of these pivotal decision points and crosstalk is a critical issue in terms of developing novel therapeutic approaches for rationalized disease management and effective patient care.

These novel therapeutic approaches may involve small molecules such as ABT-737 and HA-14A that target and inactivate anti-apoptotic Bcl-2 proteins or anti-sense Bcl-2 oligonucleotides (G3139) that target Bcl-2 at expression level to prevent the proliferation of vascular cells in the early phase of apoptosis. Moreover, utilization of peptide and non-peptide BH3 mimetics and natural or synthetic small molecules to prevent apoptosis in advanced lesion could protect against plaque destabilization and thrombin generation.

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